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Biochemistry

Purification of myosin light chain kinase from bovine cardiac muscle

(calmodulin/calcium-dependent regulator protein/calmodulin-binding protein I/calcium)

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ABSTRACT **Myosin light chain kinase was purified >100,000-fold to apparent homogeneity with a yield of 10% from bovine cardiac muscle. Sodium dodecyl sulfate gels of the purified kinase showed one stained band corresponding to a M_r of 94,000. The enzyme was activated >10-fold in the presence of Ca^{2+} (apparent K_a = 0.6–1.2 μM) and calmodulin (apparent K_a = 3–5 nM). The purified enzyme had a specific activity of 20–30 μmol of phosphate transferred per min per mg from ATP to cardiac myosin light chain 2. One mole of phosphate was incorporated per 94,000 g of the kinase in the presence of Ca^{2+} and calmodulin or of cyclic AMP-dependent protein kinase or of both additions. In addition to myosin light chain kinase, a calmodulin-binding protein of unknown function was purified from bovine cardiac muscle. This protein had a M_r of 85,000, was composed of two dissimilar subunits (M_r of 61,000 and 15,000), and competed with myosin light chain kinase for calmodulin. The protein appears to be closely related to the calmodulin-binding protein I purified from brain.**

Work from several laboratories suggested that the regulation of muscle contraction by Ca^{2+} may be mediated not only by the troponin system but also by phosphorylation of one of the light chains of myosin (light chain 2 or regulatory light chain; $M_r \approx 20{,}000$) (1, 2). Phosphorylation of light chain 2 is catalyzed by a Ca^{2+}-dependent protein kinase, referred to as myosin light chain kinase. The kinase is activated by the complex of Ca^{2+} and the calcium-dependent regulator protein (calmodulin) (3, 4). It was originally identified in skeletal muscle (5) and, later, also in cardiac and smooth muscles and in nonmuscular tissues (for a review, see ref. 2). *In vivo* phosphorylation of light chain 2 has been observed in all three muscular tissues (6–11). In addition, evidence has been presented that the contraction and relaxation of some smooth muscle preparations are regulated, at least partially, by phosphorylation and dephosphorylation of myosin light chain 2 (for a review, see ref. 12). However, the physiological significance of this reaction for the regulation of cardiac contractility is still obscure. In addition, purification of a cardiac myosin light chain kinase with a low specific activity (13) compared to the purified enzymes from skeletal and smooth muscles (14–16) has been reported. In order to define more precisely the possible components of such a myosin-linked calcium-dependent regulation system in cardiac muscle, the kinase has been purified from this tissue.

METHODS AND MATERIALS

Kinase Assay. Myosin light chain kinase activity was determined at pH 7.0 in a total volume of 50 μl containing 2 μmol of *N*-{[tris(hydroxymethyl)methyl]-2-amino}ethanesulfonic acid (Tes; Sigma), 5.0 nmol of ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.12 mg of mixed cardiac myosin light chains, 0.35 μmol of magnesium acetate, 50.0 nmol of [γ-^{32}P]ATP, 27.5 nmol of calcium chloride, 6 μg of calmodulin where indicated, and appropriate amounts of enzyme. Incubations were carried out for 2 min at 30°C. Reactions were terminated and ^{32}P incorporation into myosin light chain 2 was determined as described by Reimann *et al.* (17).

Other Methods. Polyacrylamide gel electrophoresis in the absence and presence of 0.1% $NaDodSO_4$ was carried out according to Laemmli (18). Incorporation of ^{32}P into myosin light chain kinase was measured either by the filter paper method (17) or by gel electrophoresis after boiling of the reaction mixture in the presence of 0.3% $NaDodSO_4$ and 0.5 M 2-mercaptoethanol. The gels were stained, destained, and sliced into 1-mm pieces. The pieces were dried on filter paper squares and radioactivity was measured. Values obtained by either method did not differ by more than 15%. Calmodulin was coupled to Sepharose 4B (Pharmacia) at an approximate concentration of 1 mg/ml of resin as described by Klee and Krinks (19). [γ-^{32}P]ATP was prepared according to Johnson and Walseth (20).

Purification of Proteins. Myosin light chain kinase was extracted from fresh bovine cardiac muscle and the extract was chromatographed on a DEAE-cellulose column (DE-52, Whatman) as described (21) except that all buffers contained 0.2 mM benzamidine and 0.2 mM phenylmethylsulfonyl fluoride. Those fractions that contained kinase activity and eluted around 3 mS were pooled, and benzamidine was added to a final concentration of 2 mM. The pooled fractions were then dialyzed overnight against 25 liters of 20 mM Tris·HCl, pH 7.5/0.2 mM EDTA/2 mM benzamidine/15 mM 2-mercaptoethanol. The pH of the dialyzed fractions was adjusted to 8.2 and the concentrations of NaCl, $Mg(CH_3COO)_2$, $CaCl_2$, and leupeptin (Peninsula Laboratories, San Carlos, CA) were raised to 100 mM, 2.5 mM, 2.0 mM, and 1 μg/ml, respectively. Calmodulin-Sepharose 4B (60 ml of settled resin), equilibrated in buffer A [25 mM Tris·HCl, pH 8.2/0.2 mM EDTA/2 mM benzamidine/200 mM NaCl/2.5 mM $Mg(CH_3COO)_2$/2.0 mM $CaCl_2$/1 μg of leupeptin per ml/15 mM 2-mercaptoethanol], was added and the slurry was stirred for 30 min. The resin was collected on a funnel, washed extensively with buffer A, and poured into a column. Absorbed protein was eluted by addition of buffer A containing 5 mM EGTA and no metal ions. The calmodulin-Sepharose 4B step was repeated once more with the nonabsorbed fractions. Those fractions that were eluted by EGTA and showed absorbance at 280 nm were pooled and dialyzed against 2 liters of buffer B (10 mM potassium phosphate,

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Abbreviations: Tes, *N*-{[tris(hydroxymethyl)methyl]-2-amino}ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; cAMP, cyclic AMP.

Table 1. Purification of bovine cardiac muscle myosin light chain kinase

Fraction	Protein, mg	Total activity, units	Specific activity, units/mg	Yield, %	Purification, -fold
Crude extract	140,760	20.0*	0.00014	100	1
1st DEAE-cellulose	3,740	19.8	0.0053	99	37
1st calmodulin-Sepharose	8.4	5.3	0.63	26	4,430
2nd DEAE-cellulose	0.33	2.8	8.5	14	59,800
2nd calmodulin-Sepharose	0.10	1.8	18.5	9	125,000

Activity of kinase was determined in the presence of Ca^{2+} and calmodulin. One unit equals 1 μmol of phosphate transferred to mixed myosin light chains per min.
* Activity in the absence of Ca^{2+} and calmodulin (i.e., 7.0 units) has been subtracted.

pH 6.0/0.2 mM EDTA/0.2 mM benzamidine/15 mM 2-mercaptoethanol). The dialyzed fractions were then applied to a second DEAE-cellulose column (2 ml) equilibrated with buffer B. The column was washed with 10 ml of buffer B and eluted by a linear NaCl gradient from 0 to 250 mM in 150 ml of buffer B. Fractions containing either a calmodulin-binding protein or the kinase activity were pooled as shown in Fig. 1. Both pools were concentrated on separate calmodulin-Sepharose 4B columns (2 ml) by the procedure given above. The concentrated pools were dialyzed against 10 mM Tes, pH 7.5/0.2 mM EDTA/0.2 mM benzamidine/15 mM 2-mercaptoethanol. The kinase activity and the molecular weights of the proteins, as judged by $NaDodSO_4$ gel electrophoresis, remained constant for at least 6 weeks when stored at 4°C.

Calmodulin was isolated from the first DEAE-column by a procedure adapted from that described by Sharma and Wang (22). The catalytic subunit of cyclic AMP (cAMP)-dependent protein kinase II was purified from bovine cardiac muscle as described by Beavo *et al.* (23). Myosin light chains were prepared from myofibrils of bovine cardiac muscle by the method of Perrie and Perry (24). Before use, mixed myosin light chains were chromatographed on DEAE-cellulose and were essentially free of calmodulin. Myosin light chain 2 was prepared from the total myosin light chain fraction as described by Toste and Cooke (25). Protein was determined according to Lowry *et al.* (26).

RESULTS

Purification of Cardiac Myosin Light Chain Kinase. The results of a typical preparation starting with 5.9 kg of bovine heart muscle are shown in Table 1. As noted previously (13), cardiac myosin light chain kinase was retained in the presence of calcium by a calmodulin-Sepharose 4B column. This step resulted in a large enrichment of the enzyme activity but was not specific for the cardiac kinase; several other proteins were retained in the presence of calcium by this column (19). These other proteins were separated from the cardiac kinase by ion-exchange column chromatography (Fig. 1). The purified enzyme migrated after the last purification step (i.e., the second calmodulin-Sepharose 4B column) as a single band on polyacrylamide gels in the presence (Fig. 1) and absence (results not shown) of 0.1% $NaDodSO_4$. Variation of the concentration of acrylamide from 5% to 12.5% did not reveal an additional stained band. The position of the single stained band found in the presence of $NaDodSO_4$ corresponded to a M_r of 94,000.

Kinetic Parameters of Cardiac Myosin Light Chain Kinase. Kinetic parameters were determined by using several preparations of pure enzyme, several preparations of mixed cardiac myosin light chains, or isolated myosin light chain 2. Under all conditions, radioactive phosphate was incorporated only into myosin light chain 2, as judged by $NaDodSO_4$ gel electrophoresis. Kinase activity was stimulated over 10-fold in the presence of Ca^{2+} (apparent K_a = 0.6+1.2 μM) and calmodulin (apparent K_a = 3–5 nM). The K_m for ATP was 0.22 mM and that for isolated myosin light chain 2 was between 11 and 20 μM (Fig. 2). Reciprocal transformation of the obtained values resulted in a straight line (Fig. 2). Under optimal conditions, between 20 and 30 μmol of phosphate were transferred per min per mg to myosin light chain 2 and 0.037 μmol per min

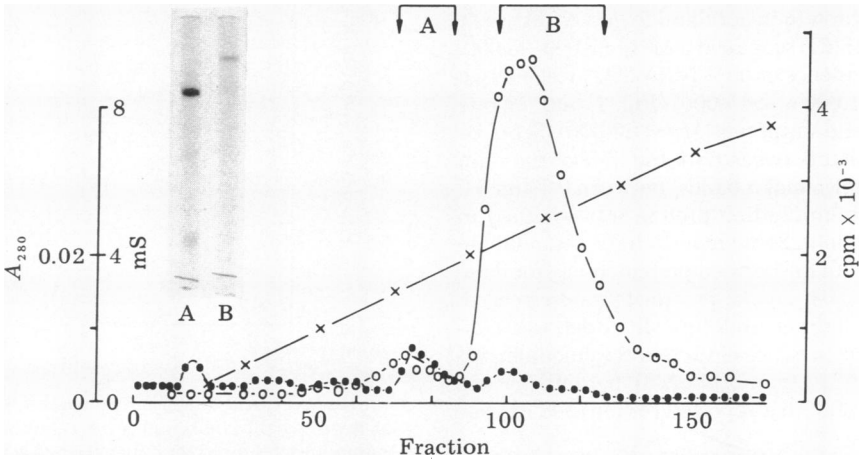

FIG. 1. Separation of a calmodulin-binding protein and myosin light chain kinase on a DEAE-cellulose column. The size of the fractions was 1 ml. The fractions labeled by arrows A and B were used for further purification of the calmodulin-binding protein and kinase, respectively. (*Inset*) 7.5% $NaDodSO_4$ gels of the final purified preparations of calmodulin-binding protein (*A*, 6 μg) and kinase (*B*, 1.3 μg). x, Conductivity in mS; ●, absorbance at 280 nm; o, kinase activity determined in the presence of Ca^{2+} and calmodulin.

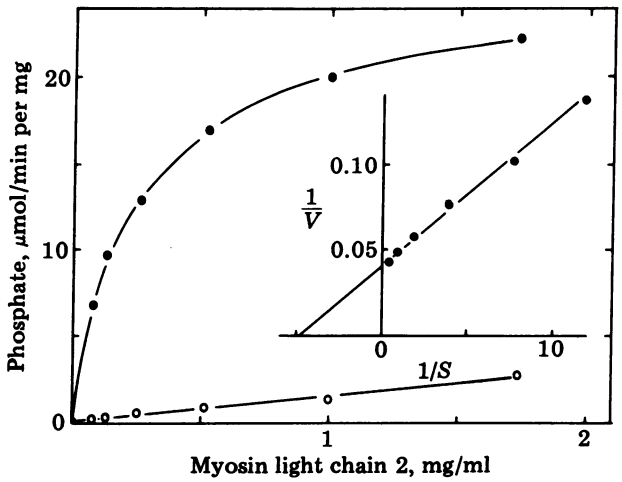

FIG. 2. Kinetics of myosin light chain 2 phosphorylation. Phosphorylation of isolated myosin light chain 2 added at the indicated concentrations was determined in the presence of Ca^{2+} and in the absence (○) and presence (●) of calmodulin. The reaction was started by addition of 30 ng of purified myosin light chain kinase. (*Inset*) Values obtained in the presence of calmodulin and Ca^{2+} and transformed according to Lineweaver and Burk.

per mg to mixed histones.*

Phosphorylation of Cardiac Myosin Light Chain Kinase. One mole of phosphate was incorporated per 94,000 g of pure kinase either in the presence of Ca^{2+} and calmodulin or in the presence of 0.28 μM catalytic subunit of cAMP-dependent protein kinase (Fig. 3). Phosphorylation of the kinase was not observed in the absence of Ca^{2+} and calmodulin. This suggested that the enzyme is phosphorylated either by itself or by the cAMP-dependent protein kinase. Simultaneous addition of Ca^{2+}, calmodulin, and catalytic subunit resulted in a slightly increased rate of phosphorylation when compared with the rates obtained in the presence of either catalytic subunit or Ca^{2+} and calmodulin. However, no significant increase in the amount of phosphate incorporated into the kinase was observed. It was demonstrated by $NaDodSO_4$ gel electrophoresis that, under all conditions, radioactive phosphate was associated only with the M_r 94,000 band of cardiac myosin light chain kinase.

Purification of a Calmodulin-Binding Protein. Fractions 68–81 of the second DEAE-cellulose column (Fig. 1) contained a protein that bound to the calmodulin-Sepharose 4B column and migrated on regular polyacrylamide gels as one band. Electrophoresis of this protein in acrylamide gels of different concentrations (5–12.5%) (27) suggested a M_r of 85,000–95,000. Electrophoresis in the presence of 0.1% $NaDodSO_4$ yielded two stained bands (M_r of 61,000 and 15,000) (Fig. 1) and, on one occasion, one minor band (apparent $M_r \approx 33,000$). Scans of stained gels indicated that between 85% and 95% of the stain was associated with the two major bands. Between 0.5 and 0.9 mg of purified calmodulin-binding protein was obtained in three different preparations. Some myosin light chain kinase activity was associated with the purified protein (range for three preparations was 0.005–0.04 μmol of phosphate transferred per min per mg to myosin light chain 2). cAMP added to a concentration of 3 mM in the presence of Ca^{2+} and calmodulin was hydrolyzed at a rate of 0.1–1.0 μmol of cAMP per min per mg, which is 1/1000th–1/100th that reported for purified cardiac phosphodiesterase (28).

* Purified myosin light chain kinase phosphorylated chicken gizzard myosin light chain 2 at a rate of about 0.4–1.5 μmol of phosphate transferred per min per mg.

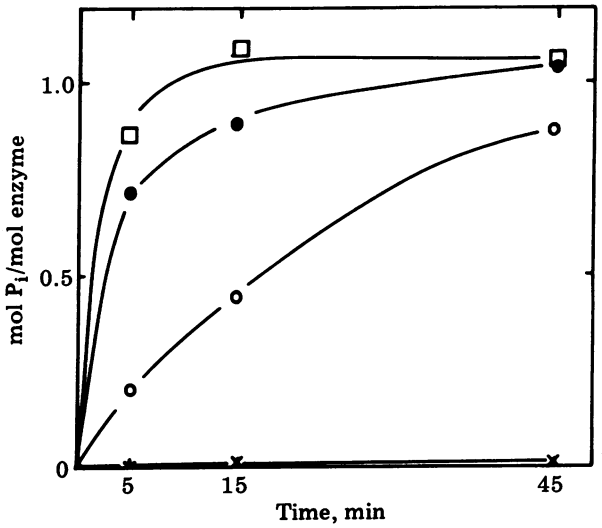

FIG. 3. Phosphorylation of bovine cardiac myosin light chain kinase. Phosphorylation of the cardiac kinase (1.3 μg) was determined in the absence of substrate proteins. All values were confirmed by the $NaDodSO_4$ method. Reactions were started by addition of [γ-^{32}P]ATP. x, Absence of Ca^{2+} and calmodulin, presence of 28 nmol of EGTA; ○, absence of Ca^{2+} and calmodulin, presence of 0.28 μM pure catalytic subunit of cAMP-dependent protein kinase and 28 nmol of EGTA; ●, presence of Ca^{2+} and calmodulin alone; □, presence of Ca^{2+}, calmodulin, and catalytic subunit.

Interference of Calmodulin-Binding Protein with Myosin Light Chain Kinase Activity. The purified calmodulin-binding protein reduced the activity of pure myosin light chain kinase when the activity of the kinase was determined at nonsaturating concentrations of calmodulin. This inhibition could be prevented by increasing the concentration of calmodulin (Fig. 4). These results indicated that the kinase and the calmodulin-binding protein competed for the complex of calmodulin and Ca^{2+}.

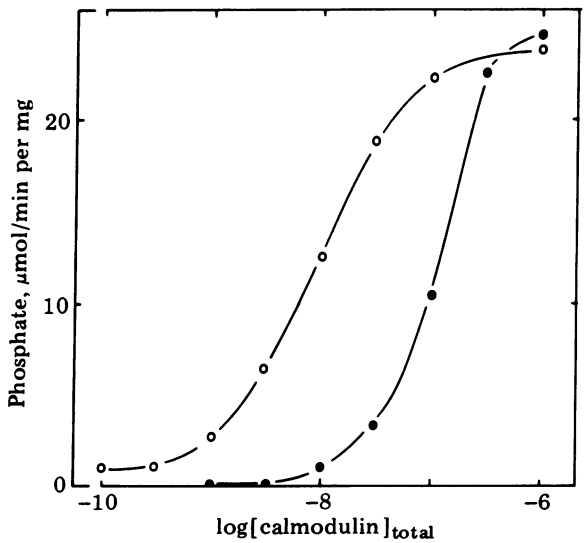

FIG. 4. Effect of cardiac calmodulin-binding protein on activation of myosin light chain kinase by calmodulin. The concentration of calmodulin required for half-maximal activation of the pure cardiac kinase (10 ng) was determined in the absence (○) and presence (●) of 0.6 μg of purified cardiac calmodulin-binding protein. Addition of boiled calmodulin-binding protein had no effect on the concentration of calmodulin required for half-maximal activation of the kinase.

DISCUSSION

A calmodulin-binding protein and a kinase that phosphorylates myosin light chain 2 with high velocity have been purified to apparent homogeneity from bovine cardiac muscle. The molecular weights of the calmodulin-binding protein and of its subunits are almost identical to those of calmodulin-binding protein I (29), also referred to as "calcineurin" (30), which has been detected at high concentrations (52 mg/kg wet weight) in bovine brain. This and other similarities (e.g., its heat lability and its ability to interfere with activiation of cardiac myosin light chain kinase by calmodulin) suggest that the cardiac protein is closely related to or identical with the brain protein. However, the identity of the cardiac calmodulin-binding protein with the brain protein remains to be established.

Purified cardiac muscle myosin light chain kinase, as purified in this study, has properties similar to those of the enzymes purified from skeletal (14) or smooth muscle (15, 16). All three enzymes have a high turnover number when myosin light chain 2 phosphorylation is measured and need millimolar concentrations of ATP for optimal activity. The minimal M_r of the cardiac kinase (94,000) is similar to that reported for the skeletal muscle enzyme (80,000–93,000) (14, 28), but is lower than that reported for a smooth muscle enzyme (125,000) (16). The cardiac kinase is phosphorylated in the presence of Ca^{2+} and calmodulin, suggesting a self-phosphorylation reaction. Like one enzyme from smooth muscle (16), cAMP-dependent protein kinase also phosphorylates cardiac myosin light chain kinase *in vitro*, although at a rather slow rate. Therefore, the physiological significance of the latter reaction is doubtful. In addition, no functional change correlated with the incorporation of 1 mole of phosphate has so far been detected (unpublished observation). Some of the results obtained in this study (e.g., specific activities and phosphorylation by itself or with cAMP-dependent protein kinase) are at variance with a previous report on the purified cardiac kinase (13). The reason for the differences are unclear, but may be related to differences in proteolytic activities present during the purification. The high specific activity of cardiac myosin light chain kinase obtained in this study suggests that phosphorylation of cardiac myosin light chain 2 may be of physiological significance.

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